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Analysis of expression of an alternative La (SS-B) cDNA and localization of the encoded N- and C-terminal peptides

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Abstract

A deletion of an (A)-residue was detected in a cDNA encoding for the nuclear autoantigen La/SS-B. The cDNA was recently isolated from a cDNA library made from peripheral blood lymphocytes of a patient with primary Sjögren's Syndrome. The region, where the deletion occurred, represents a hot spot region in the La gene(s). It leads to a frame shift mutation and a premature stop codon eleven amino acids downstream of the deletion site within one of the protease sensitive regions of the La protein. In spite of the frame shift mutation expression of full length La protein occurred efficiently in *E. coli*. Full length La protein was also made in SF9 cells infected with recombinant baculoviruses, although the efficiency of full length protein production was less. Two major peptides with molecular weights of 29 kDa and 25 kDa were made. The size of these peptides was similar to the known proteolytic degradation products of La protein. The N-terminal 29 kDa fragment containing the RNP consensus sequence located in the cytoplasm. The 25 kDa C-terminal fragment containing the nuclear location signal entered in the nucleus and associated with nuclear speckles. In conclusion, the ability to (i) enter, (ii) remain in the nucleus and (iii) assemble with nuclear speckles resides in the C-terminal domain of La protein and does not depend on the N-terminal RNP-consensus motif.

1. Introduction

Sera of patients with systemic immuno-inflammatory diseases such as Systemic Lupus Erythematosus (SLE) or primary Sjögrens Syndrome (pSS) frequently contain autoantibodies to nuclear antigens [1]. The La (also known as SS-B) antigen constitutes one of the targets of such anti-nuclear antibodies (ANAs). La protein was found to associate with nearly all RNA polymerase III transcripts at least transiently as well as with a series of leader RNAs of different viruses [2,3]. The La protein was described as a protein with a molecular weight of about 50 kDa. It consists of two domains which are connected by an α-helical stretch that is very sensitive to proteolysis. Cleavage of La protein results in the release of a N-terminal 29 kDa and a C-terminal 25 kDa fragment [4]. The N-terminal fragment contains a RNP-motif, that has been shown to be both, required and sufficient for RNA binding [5,6]. The C-terminal fragment was determined to start at the amino acid (aa) methionine 223 (M223) [4]. It contains two regions similar to sequence elements of double-stranded RNA-activated protein kinase PKR, the nuclear location signal (NLS) and a putative ATP-binding site [7–9].

A variety of staining pattern had been reported in the literature for anti-La antibodies including a nu-

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clear speckled type pattern, a homogenous nuclear staining, a cytoplasmic staining, and a staining of both compartments [summarized in [10]]. In some cases, especially after virus infections a nucleocytoplasmic translocation was reported including infection with herpes simplex virus type I [10] and poliovirus [11]. Moreover, a leakage of La protein during fixation was suggested to be responsible for the cytoplasmic staining of anti-La antibodies.

La protein was assumed to be involved in transcription termination of RNA polymerase III [12,13]. In addition, La protein was proposed to be involved in internal initiation of translation at least in poliovirus infected cells [11].

Most recently five La cDNAs were isolated, when a cDNA library made from peripheral blood lymphocytes (PBL) of a patient with pSS was screened with her own anti-La serum [14]. In two of these five La cDNAs the exon 1 was replaced with an alternative exon termed exon 1′.

Here we report that one of the two exon 1′ La cDNAs contained a deletion of an (A)-residue in the protease sensitive peptide region which connects the two domains of the La protein. In spite of the frame shift mutation full length recombinant La protein was efficiently made when the deletion mutant was expressed in *E. coli*. Furthermore, when a recombinant baculovirus containing the mutated coding region of La protein was expressed in SF9 cells the baculovirus also allowed the expression of full length protein. However, the full length La protein expression was less efficient and the majority of La protein was fragmented to the N- and C-terminal La protein domain. The N-terminus remained in the cytoplasm, the C-terminus entered in the nucleus and assembled with nuclear speckles.

2. Materials and methods

2.1. Materials

*Ava*II, *Bam*HI, *Bst*EII, *Kpn*I, *Xho*I, and T7-Sequencing kit were obtained from Pharmacia (Freiburg, Germany); *Nco*I from MBI Fermentas (St. Leon-Rot, Germany); pQE-60W, QIAprep-spin kit and QIAEX from Qiagen (Hilden, Germany); T.C. 100 medium, Lipofectin and Taq polymerase from Gibco BRL Life Technologies (Eggenstein, Germany); pET-3d (AGS, Heidelberg, Germany); pBluescript SK(−) from Stratagene GmbH (Heidelberg, Germany); CDP-Star TRopix, and pGEM-T vector systems from Promega (Serva, Heidelberg, Germany); Baculogold DNA, and pVL 1393 from Dianova (Hamburg, Germany); *Bst*XI, *Hin*dIII, *Taq*-buffer (10 ×), DNA molecular weight marker VI, positively charged nylon membrane (1209272), and blocking reagent from Boehringer Mannheim (Mannheim, Germany); agarose and NuSieve agarose from Biozym (Hameln, Germany); anti-mouse IgG conjugated with peroxidase developed in sheep ($(Fab')_2$ fragments) adsorbed with human serum proteins (A-7282; 20 U/ml), anti-human IgG (γ-chain specific) conjugated with alkaline phosphatase (A3150; 8 U/ml), and isopropyl β-D-thiogalactopyranoside (IPTG) from Sigma (St. Louis, MA, USA); the ECL-Western blotting detection reagents were from Amersham-Buchler (Braunschweig, Germany); 4-Nitro-blue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) from Roth (Karlsruhe, Germany); PVDF-membrane (IPVH 000 10; pore size 0.45 μm) was obtained from Millipore (Bedford, MA, USA).

2.2. Monoclonal antibodies and sera

Two monoclonal anti-La antibodies (anti-La mAb) and a patient anti-La antibody were used. The anti-La mAb La4B6 was elicited by immunization of Balb/C An mice with recombinant human La protein. The epitope recognized by the anti-La mAb La4B6 was shown to be the aa sequence ^{331}SKGRRFKGKGKGN^{343}. This region includes the putative ATP-binding site of the La protein within the C-terminal domain [9]. The anti-La mAb SW5 was originally described by Smith et al. [15]. It was shown to be directed to the N-terminal domain of La protein [16] and kindly provided by Prof. Dr. van Venrooij (University of Nijmegen, The Netherlands). The patient anti-La antibody was obtained from the serum of the patient (Ma) [13]. It was immunoadsorbed to recombinant La protein.

2.3. Cell culture and staining procedures

SF9 cells were grown in T.C. 100 medium containing 10% FCS to a density of about 10^6 cells/ml.

Then the cells were infected with 10 pfu/cell of the respective recombinant baculovirus (see below). 40 to 50 h p.i. the cells were harvested for the preparation of extracts (see below). Prior to the preparation of extracts 100 μl aliquots of infected or uninfected SF9 cells were taken and centrifuged to coverslips at $200 \times g$ for 2 min at room temperature using a Cytofuge (Heraeus, Hanau, Germany).

The cells were fixed with methanol containing 0.02% EGTA at -20°C for 1 h. Prior to immunostaining the cells were rehydrated for 5 min with PBS. Then the cells were incubated with cell culture supernatant of hybridomas secreting the respective anti-La mAb for at least 15 min. The cells were washed with PBS (5 min) and the bound anti-La mAb was detected using Cy3-conjugated anti-mouse antibody developed in goat. Indirect immunofluorescence labeling using immunoadsorbed patient anti-La antibody was performed using the same procedure. The bound human anti-La antibody was detected with species-specific anti-human antibody conjugated with Fluorescein (FITC). For double immunofluorescence, the cells were first stained with the respective anti-La mAb and the anti-mouse antibody conjugated with the fluorescent dye Cy3 as described above. Then the cells were additionally incubated with the patient anti-La antibody. The detection of the bound human autoantibody was performed using anti-human antibody conjugated with FITC. In all cases the incubation time for the secondary antibody was 15 min. The staining occurred at room temperature. The unbound secondary antibodies were removed by washing with PBS (twice, 5 min). The stained cells were mounted using PBS/glycerol (1:1 [v/v]).

2.4. Preparation of extracts

Total extracts were prepared from uninfected SF9 cells or SF9 cells which were infected with either wildtype baculovirus or the respective recombinant baculovirus (see below). 40 ml cell suspension was harvested by centrifugation ($14\,000 \times g$ for 5 min at 4°C), resuspended in 1.4 ml of cell lysis buffer, heated for 5 min to 95°C and centrifuged at $14\,000 \times g$ for 5 min at 4°C. 5 μl aliquots were mixed with SDS-PAGE sample solution [17] and used for SDS-PAGE.

2.5. SDS-polyacrylamide gel electrophoresis and immunoblotting

SDS-PAGE was performed according to Laemmli and immunoblotting to PVDF membrane according to Matsudaira [17,18]. After blocking and washing the blots were incubated with cell culture supernatant

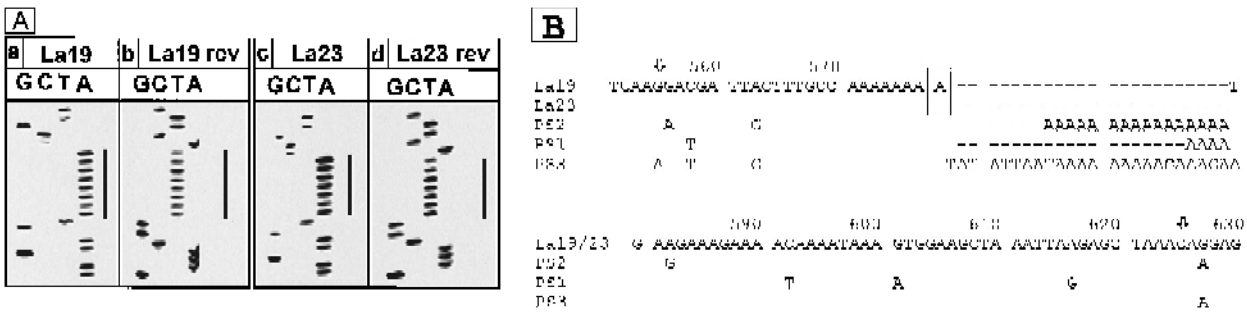

Fig. 1. Comparison of the exon 7 sequence of the La cDNAs La19 and La23. (A) Identification of a frame shift mutation in the La cDNA La23 (lanes c,d) but not in the La cDNA La19 (lanes a,b). The interesting region was sequenced from both orientations (rev = reversed). The bars indicate the localization of the correct oligo$(A)_8$ fragment in the exon 7 in the La19 cDNA (lanes a,b) and the localization of the deleted oligo$(A)_7$ fragment in La23 (lanes c,d). (B) Comparison of the nt sequences of La19 (EMBL Acc. Nr. X69804), La23 and the La pseudogenes (PS1 to 3; (EMBL Acc. Nrs. X91336; X91337; X91338)). The box shows the lack of a single (A)-residue in La23 compared to La19. (−) indicates the lack of a nt in the respective sequence compared to the longest insert in the exon 7 of PS3. The arrows mark the start and end of exon 7.

of hybridoma cells secreting the anti-La mAb La4B6 or SW5. Formed immune complexes were visualized using the enhanced chemiluminescence (ECL)-Western blotting detection reagents. Then the immune complexes were eluted and the blot was incubated with the human anti-La serum. The formed immune complexes were detected using anti-human antibodies conjugated with alkaline phosphatase and BCIP/NBT as substrate. In case that the blot was analyzed with the anti-La mAb SW5 followed by the anti-La mAb La4B6 the second detection was also performed with the ECL system.

2.6. Confocal laser scanning microscopy

Confocal laser scanning microscopy (cLSM) was performed using a Zeiss LSM 10. The stained specimens were cut automatically into horizontal sections (512×512 pixels/8 bit, objective lenses Plan-Neofluar $40 \times /1.3$ oil). Evaluation of the stored stacks of the horizontal optical sections was performed with the LSM 10 image processing unit.

2.7. DNA sequence analysis

DNA was prepared using the QIAprep-spin kit and concentrated by ethanol precipitation to a final concentration of about 1 μg/μl in TE buffer [19]. DNA sequencing was performed as described previously [14].

2.8. Construction of the recombinant baculoviruses

Recently two La cDNAs, which were obtained by immunoscreening of a cDNA library of PBL of a patient with pSS with the patient's own autoimmune serum, were characterized (La19/La23; EMBL Acc. Nr. X69804; [14]). The clone La23 contained a deletion of an (A)-residue in the protease sensitive region between the N- and C-terminal fragment (Fig. 1). This La cDNA was used to construct a recombinant baculovirus according to the following strategy: the core portion of the La cDNA (spanning from the *Kpn*I site to the *Ava*II site) was isolated. The 5′- and 3′-portions were modified by use of a PCR technique. For the 3′-portion we used as the upstream primer P1 (P1:CGAAATTTGCTAGTGATGATGAACA) and as downstream primer P2 (P2:TGGTTTGGATCCCTACTGGTCTCCAG; the artificial *Bam*HI site neighbouring the stop codon TAG (CTA) is underlined). The resulting fragment was cut by *Ava*II and *Bam*HI and subcloned into pBluescript SK(−). The 5′-end was created as follows.

In the regular La mRNA form translation starts at the first AUG locating in the exon 2. Such a 5′-terminal construct was prepared from the cDNA La23 by PCR using the primer P3 (P3:ACATAGGATCCATGGCTGAAAATGGT; the artificial *Bam*HI site neighbouring the translational start ATG is underlined) as upstream primer and P4 (P4:TGTTGTTAGACGGTTCAACCTGTTG) as downstream primer. The PCR fragment was cleaved by *Bam*HI and *Kpn*I and cloned into the corresponding sites of pBluescript SK(−) resulting in the construct pBSLa (pBluescript containing the La coding region).

In order to express the La protein reading frame in *E. coli* pBSLa was cut with *Nco*I/*Bam*HI and cloned into the respective cloning sites of pQE-60W. Thereby a C-terminally His-tagged La protein construct was obtained. As the expression of His-tagged La protein from this construct was low, it was further subcloned into the expression vector pET-3d using the *Nco*I/*Hin*dIII sites. This construct expressed C-terminally His-tagged La protein (Fig. 3).

The constructs for cloning into pVL 1393 were rearranged using the different portions after cleavage by *Kpn*I, *Bam*HI and *Ava*II. The whole molecules were cloned into the *Bam*HI site of pVL1393.

The cotransfection for the production of the infectious recombinant baculovirus was performed as follows. First a mixture consisting of 0.4 μg of the construct, 0.1 μg Baculogold DNA, and 30 μg Lipofectin within 50 μl of distilled water was prepared in polysterene tubes. After 15 min at room temperature 1 ml T.C. 100 medium without FCS was added and transferred to 6×10^5 SF9 cells seeded in a Petri dish ($d = 3$ cm) and incubated for 4 h at 27°C. Then 1 ml T.C. 100 medium containing 10% FCS was added. After seven days the supernatant containing the recombinant viruses was harvested by centrifugation ($14\,000 \times g$ for 15 min at room temperature) and used for further amplification of the virus titer. Stock solutions were prepared and stored at 4°C for infection and expression of the respective La proteins.

3. Results

3.1. Identification of a frame shift mutation in the exon 7 of the La cDNA La23

When the La cDNA La19 was used for sequencing an oligo(A) stretch of 8 (A)-residues was detectable in the exon 7 starting at nt position 1054 (Fig. 1A,a,b). The same oligo(A)-stretch had also been reported in a series of other human La cDNAs (EMBL Acc. Nrs. X13697; R20464; M20328; J04205; M11108; M35263). In contrast, the La cDNA La23 showed a deletion of an (A)-residue in this oligo(A)-stretch (Fig. 1 A, c,d). The different lengths of the oligo(A)-stretches in the two La cDNAs were confirmed when sequencing was performed from both orientations. The deletion located in the protease sensitive region, where La protein can be cleaved resulting in the 29 kDa N-terminal and 25 kDa C-terminal fragment [4]. Interestingly, the deletion located at the same site, where the three La pseudogenes (EMBL Acc. Nrs. X91336; X91337; X91338) contained more or less homogenous oligo(A) inserts (Fig. 1 B).

3.2. The frame shift mutation in the coding sequence of the La cDNA La23 is efficiently corrected by a ribosomal frame shift in E. coli

The deletion altered the reading frame in the La mRNA derived from the La cDNA La23 and leads to a premature stop codon 11 amino acids (aa) downstream of the frame shift site. Therefore one would expect that translation should cause the addition of the 11 aa (MKKENKIKWKLN) to the C-terminus of the N-terminal domain of La protein. Downstream of these aa translation should stop at the following first in frame stop codon (Fig. 2 (*)). The resulting translational product should have a molecular weight of about 29 kDa similar to the N-terminal proteolytical degradation product.

However, when the pET-3d construct, which contained only the complete mutant La protein coding region was expressed in *E. coli* (Fig. 3 A), a protein of about 50 kDa similar in size to full length La protein was made (Fig. 3 A, lanes a to h). As shown in Fig. 3 (A, lanes a,b) a monospecific anti-La antibody adsorbed to recombinant La protein reacted with this protein. The blot was eluted and cut into two stripes. One stripe was analyzed using the anti-La mAb SW5. The anti-La mAb SW5 is directed to an epitope locating upstream of the frame shift mutation. As shown in Fig. 3 A (lane d) the anti-La mAb SW5 also reacted with the 50 kDa protein. The second stripe was analyzed with the anti-La mAb La4B6, which recognizes an epitope, that locates downstream of the frame shift mutation. As shown in Fig. 3 A (lane c) the anti-La mAb La4B6 also reacted with the 50 kDa protein. In addition to the full length La protein the patient anti-La antibody reacted with two protein fragments of 29 and 25 kDa (Fig. 3 A, lanes a,b). In agreement with earlier studies, the reactivity

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H/P 160 S  I  E  S  A  K  K  F  V  E  T  P  G  Q  K  Y  K  E  T  D    179
H/P 961 AGCATTGAATCTGCTAAGAAATTTGTAGAGACCCCTGGCCAGAAGTACAAAGAAACAGAC 1020

P   180 L  L  I  L  F  K  D  D  Y  F  A  K  K  M  K  K  E  N  K  I   199
P  1021 CTGCTAATACTTTTCAAGGACGATTACTTTGCCAAAAAA-ATGAAGAAAGAAAACAAAAT 1079
H  1021                                      A                       1080
H   180 L  L  I  L  F  K  D  D  Y  F  A  K  K  N  E  E  R  K  Q  N   199

P   200  K  W  K  L  N  *                                            204
P  1080 AAAGTGGAAGCTAAATTAAGAGCTAAACAGGAGCAAGAAGCAAAACAAAAGTTAGAAGAA 1139
H  1081                                                             1140
H   200 K  V  E  A  K  L  R  A  K  Q  E  Q  E  A  K  Q  K  L  E  E   219


P   223          M  K  S  L  E  E  K  I  G  C  L  L  K  F  S  G  D   239
P  1140 GATGCTGAAATGAAATCTCTAGAAGAAAAGATTGGATGCTTGCTGAAATTTTCGGGTGAT 1199
H  1141                                                             1200
H   220 D  A  E  M  K  S  L  E  E  K  I  G  C  L  L  K  F  S  G  D   239
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Fig. 2. Consequences of the frame shift mutation in the La cDNA La23. Partial nt sequence of La19 (H, 961–1200) and La23 (P, 961–1199) and the deduced partial aa sequence of La19 (H, 160–239) and La23 (P, 160–205, 223–239).

to the C-terminal fragment was less efficient and only detectable when overexposing the blot [9]. The 29 kDa band was also detected by the anti-La mAb SW5 (Fig. 3 A, lane d). In contrast the anti-La mAb SW5 did not react with the 25 kDa band. Vice versa the anti-La mAb La4B6 reacted with the C-terminal fragment of about 25 kDa (Fig. 3 A, lane c), but not with the 29 kDa N-terminal fragment. When the total extract containing (i) full length La protein being reactive with the anti-La mAb SW5 (Fig. 3 A, lane g) and the anti-La mAb La4B6 (Fig. 3 A, lane e), (ii) the N-terminal (Fig. 3 A, lane g), and (iii) the C-terminal fragment (Fig. 3 A, lane e), were passed through an Nickel affinity column the full length La protein could be detected in the eluate of the column with both, the anti-La mAb SW5 (Fig. 3 A, lane h) and the anti-La mAb La4B6 (Fig. 3 A, lane f). Moreover, in addition to the full length La protein, the C-terminal fragment was also eluted from the column and detectable with the anti-La mAb La4B6 (Fig. 3 A, lane f). In contrast, the not-tagged N-terminal La protein fragment was removed during this purification step (Fig. 3 A, lane h).

From these results it is obvious that the His-tag and the two C-terminal locating La epitopes recognized by either the anti-La mAb La4B6 or the patient anti-La antibody located in the reading frame of the N-terminal La protein fragment. Thus the 50 kDa protein expressed from the mutant La cDNA represented full length La protein. In consequence, the region around the deletion must contain an efficient ribosomal frame shift site.

3.3. The ribosomal frame shift event occurring in E. coli also occurs in SF9 cells infected with a recombinant baculovirus

In the next step the coding region containing the deletion was used to construct a recombinant baculovirus. As shown in Fig. 3 (B, lanes o,q) extracts obtained from SF9 cells infected with this baculovirus indeed contained full length La protein. The full length La protein reacted with both, the anti-La mAb SW5 (Fig. 3 B, lane o) and the anti-La mAb La4B6 (Fig. 3 B, lane q). However, the amount of full length protein was less if compared to the bacterial expression. The SF9 cell extracts contained predominantly the N- and C-terminal La protein fragments. The N-terminal La protein fragment was reactive with both, the anti-La mAb SW5 (Fig. 3 B, lane o) and the patient's anti-La antibody (data not shown). The C-terminal fragment reacted with the anti-La mAb La4B6 (Fig. 3, lane q). Extracts obtained from SF9 cells infected with wild-type baculoviruses did not react with the anti-La mAbs (Fig. 3B, lanes k,n,p,r). In order to rule out that the N- and C-termi-

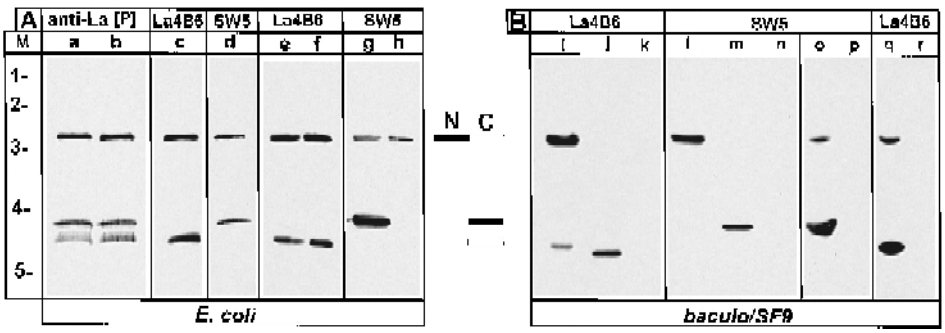

Fig. 3. Protein products expressed from the frame shift mutated La mRNA in *E. coli* or the SF9/baculovirus system. *E. coli* extracts (A, lanes a to h). SF9/baculovirus extracts (B, lanes i to r). The extracts were either detected with the patient anti-La antibody (lanes a,b), or the anti-La mAb SW5 (lanes d,g,h,l to p), or the anti-La mAb La4b6 (lanes c,e,f,i to k,q,r). Lane M: 1 to 5 positions of marker proteins. 1 = 97.5 kDa, 2 = 66 kDa, 3 = 45 kDa, 4 = 31 kDa, 5 = 21.5 kDa. (A) (lanes c,d) same blot as (lanes a,b); (lanes e,f) same blot as (lanes g,h); total *E. coli* extracts (lanes a to e,g); eluates from a nickel affinity column (lanes f,h). (B) SF9 cell extract of cells infected with recombinant baculoviruses expressing the correct La protein reading frame (lanes i,l); SF9 cell extract of cells infected with recombinant baculoviruses expressing the mutant La protein reading frame (lanes j,m,o,q); SF9 cell extract of cells infected with wildtype baculoviruses (lanes k,n,p,r). In order to identify full length La protein in the extract analyzed in lanes (j,m) the gel had to be overloaded in lanes (o,q).

nal fragments were the results of a proteolytical degradation, SF9 cells were infected with a recombinant baculovirus containing the correct La protein reading frame (Fig. 3 B, lanes i,l). In parallel SF9 cells were infected with the mutant recombinant baculovirus (Fig. 3 B, lanes j,m). As shown in Fig. 3 B (lanes i,l) the recombinant baculovirus containing the correct La protein frame resulted predominantly in an expression of full length La protein (Fig. 3 B, lanes l,i). The full length La protein reacted with both, the anti-La mAb SW5 (Fig. 3 B, lane l) and the anti-La mAb La4B6 (Fig. 3B, lane i). Only a minor amount of an additional C-terminal La protein fragment was detected with the anti-La mAb La4B6, while no N-terminal proteolytical fragment was detectable under the used conditions. No full length La protein was detectable in the extract of the SF9 cells infected with the recombinant mutant La protein (Fig. 3B, lanes j,m). Only the N- (Fig. 3 B, lane m) and the C-terminal (Fig. 3B, lane j) La protein fragments were detected. Due to lack of a His-tag the C-terminal fragment of the recombinant mutant baculovirus (Fig. 3B, lane j) had a slightly higher mobility than the C-terminal fragment of the recombinant baculovirus which contained a C-terminal His-tag (Fig. 3B, lane i).

From these results we concluded that the frame shift can also occur in the baculo/SF9 cell system, but the majority of La protein existed as two fragments, the N-terminal 29 kDa and the C-terminal 25 kDa fragment.

3.4. Localization of the N- and C-terminal La protein fragment in SF9 cells

As the N-terminal fragment contains the RNP consensus sequence and the C-terminal fragment contains the putative nuclear localization signals and ATP-binding site, we looked for the localization of the two La protein fragments in SF9 cells infected with the recombinant baculovirus. The cells were either stained with the anti-La mAb La4B6 (Fig. 4a), the anti-La mAb SW5 (Fig. 4c,d) or the patient's anti-La antibody (Fig. 4b). As shown in Fig. 4 the patient's anti-La antibody (Fig. 4b) and the anti-La mAb SW5 (Fig. 4c,d) stained predominantly the cytoplasm of SF9 cells. In contrast, the anti-La mAb La4B6 stained predominantly the nucleus (Fig. 4a)

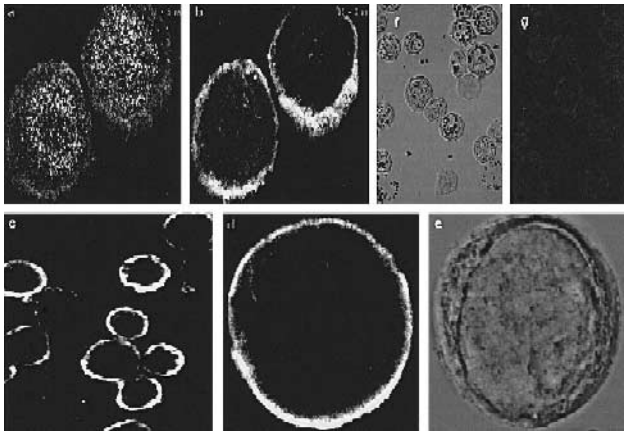

Fig. 4. Detection of La peptides in SF9 cells. SF9 cells infected with wildtype baculoviruses (f,g) stained with a mixture of all used primary and secondary antibodies, overlay of the FITC and RITC image (g). SF9 cells infected with the recombinant mutant La baculovirus (a to e). (a,b) double immunofluorescence of the anti-La mAb La4B6 (a) with the patient's anti-La antibody (b). (a,b) are overlays of ten optical sections obtained with cLSM. (c,d) staining pattern of the anti-La mAb SW5. (e,f) corresponding phase contrast images.

and gave a nuclear speckled type pattern. A similar nuclear speckled type pattern is usually found for all the anti-La antibodies including the SW5, the patient's anti-La antibody and the La4B6 on cultured cells [9].

From these results we concluded, that (i) the N-terminal fragment was not able to enter in the nucleus of SF9 cells, (ii) the C-terminus contained the nuclear location signal, (iii) the N-terminal RNP-consensus motif was not essential for assembly of La protein with nuclear speckles and, (iv) the cytoplasmic staining of the anti-La antibodies was not due to a leakage of La protein at least under these conditions.

4. Discussion

Frequently sera of patients with pSS or SLE contain self-reacting antibodies directed to nuclear antigens. One of the targets of such ANAs is the nuclear autoantigen La/SS-B [1]. It was proposed to be involved in transcription/termination of RNA polymerase III [12,13] and in internal initiation of translation especially of (polio)virus mRNAs [11].

In a recent study we searched for alternative La mRNAs by screening of a cDNA library made from

PBL of a patient with pSS with her own anti-La serum. Thereby five La cDNAs were isolated. Among them two La cDNAs (La19, La23; EMBL Acc.Nr. X69804) were found, in which the exon 1 was replaced with the alternative exon 1′. Analysis of the La gene showed that the transcription of the alternative La mRNA started in the intron between the exons 1 and 2 using a promoter switch. The resulting transcript was processed using an alternative splicing pathway.

The structure of the exon 1′ La mRNA was unusual as it contains a GC-rich 5′-terminus, three staggered ORFs upstream of the La protein reading frame and an oligo(U)-tail of 23 (U)-residues. In order to analyze the function a series of constructs were made. During these cloning steps we noticed that the La cDNA La23 contained a deletion of an (A)-residue in the coding region. The frame shift mutation located within the exon 7. During discussion of our data we were told that a similar La cDNA was isolated from a human liver cDNA library. This La cDNA contained an insert of a single (A)-residue at the same position (Dr. Haubruck, Dr. Stahnke, Fa. ELIAS, Freiburg, Germany, personal communication). When the genomic DNA of the pSS patient was analyzed no mutation was detected (data not shown). Thus, the frame shift mutation in the patient's La cDNA was either a mistake of reversed transcriptase during preparation of the La cDNA library or the result of a somatic mutation. As the cDNA library was prepared from PBL the mutation had to be expected in one of the B- or T-cells, or the macrophages of the patient.

Most recently we characterized and sequenced the three La pseudogenes (EMBL Acc. Nrs. X91336; X91337; X91338; to be published). The pseudogenes represented retrogenes as they lacked introns. Moreover, inverted repeats were found flanking the pseudogene sequences. The retrogenes were termed PS1 to PS3. In PS1 and PS3 exon 1 La mRNAs were used as templates of the La cDNAs. PS2 was the reversed transcript of an alternative exon 1′ La mRNA. Based on the total amount of mutations in the respective pseudogene we calculated the evolutionary age with 4 (PS1), 4.5 (PS2) and 5 (PS3) million years. The calculation was performed according to Lin and Chan [20].

During sequence analysis of the retrogenes we noticed that the frame shift mutation in the La cDNAs located just at the same position where in all the three La pseudogenes inserts of more or less long oligo(A)-stretches of different lengthes ranging from 4 (PS1) to 16 (PS2) to 24 nts (PS3) occurred in addition to an oligo$(A)_8$-stretch. In consequence, the oldest pseudogene contained the longest oligo$(A)_{32}$-stretch, while the youngest pseudogene contained the shortest oligo$(A)_{12}$-stretch in the exon 7.

As in the case of the deletion/insert in the La cDNAs, the inserts of the (A)-residues in the retropseudogenes could be explained by the same mechanisms. They were the result of either mistakes which occurred during reversed transcription or somatic mutations in a hot spot region in the exon 7 of the La gene and pseudogenes. Mistakes made by reversed transcriptase appeared to be rather unlikely for several reasons. Until now more than 30 human La cDNAs had been isolated from independent groups [4,22–26]. Only in the two La cDNAs frame shift mutations were found in the exon 7. Therefore, it appears rather unlikely that reverse transcriptase has inserted up to 24 (A)-residues in just all those three transcripts which were then inserted as retrogenes in the human genome. Moreover, based on the age of the individual retrogene it is obvious that the oldest pseudogene (PS3) contained the longest insert (24 residues), while the youngest pseudogene (PS1) contained the shortest insert (4 (A)-residues). As homopolymeric sequence increase the probability of recombinations and thereby decrease the stability of a genome, homopolymeric sequences especially the 3′-poly(A)-tails of pseudogenes are usually rapidly deleted or mutated. Therefore, one would expect, that the length of the oligo(A)-stretches in the exon 7 will be mutated and lost during further evolution, but should not increase. This assumption is in good agreement with the following observation. As mentioned above PS2 is a derivative of an alternative exon 1′ La mRNA. Thus the exon 1′ La mRNA which was used as template for the retrogene contained a further homopolymeric oligo(U)-stretch of 23 (uridine) residues. When looking to the exon 1′ sequence of PS2, it is obvious that this oligo(dT)-stretch was partially deleted and mutated. Thus the evolutionary time was enough to mutate this oligo$(dT/dA)_{23}$-stretch in the 5′-exon 1′. In contrast, the oligo$(dA/dT)_{24}$-stretch in the exon 7 was con-

served in the same pseudogene without any selection pressure. Therefore, from our point of view, it appears more likely that the already existing oligo(A)-stretch of 8 (A)-residues in the human La gene (and 9 in mouse and rat [21]) and the further increase of length of this oligo(A)-stretch in dependence on the age of the pseudogenes is caused by an repeated insert of (A)-residues but not the result of reversed transcriptase mistakes. As an insert in the La gene interfered with its function the frequency of an efficient insert in the La gene was less than in the pseudogenes. Taken together, the exon 7 might represent a hot spot region in the La gene and pseudogenes, where repeated inserts of (A)-residues can occur. This is also of interest, as one of the major autoepitopes of the La antigen was located in the hot spot region [22].

In consequence, the frame shift mutations in the two La cDNAs located in a hot spot region of the La genes. Therefore, it is also likely that the corresponding template La mRNAs already contained these mistakes. Thus they were transcripts from La genes in a cell containing the respective somatic mutation.

Based on the deletion in the reading frame, we expected that an expression of this La cDNA should provide us with the N-terminal 29 kDa fragment of La protein containing the RNP consensus sequence. For this purpose the La coding region containing the frame shift mutation was cloned in the pQE-60W vector. During this cloning step a C-terminal oligo(His)-tag was introduced in the reading frame of La protein, which should allow us to remove C-terminal La protein fragments when formed by internal initiation. Unfortunately, this construct preferentially expressed the C-terminal fragment indicating that an internal translation initiation occurred downstream of the frameshift site (data not shown). The only AUG which located (i) in the reading frame of La protein and (ii) downstream of the frame shift site located at aa position (M(223); Fig. 2). Indeed, already during epitope mapping of the anti-La mAb La4B6 this M(223) was suggested to be involved in internal initiation of translation. This conclusion based on the following observation. The epitope recognized by the anti-La mAb La4B6 (SKGRRFKGKGKGN) locates downstream of M(223). As the reactivity of the anti-La mAb to *E. coli* extracts, which contained La peptides expressed from 5′-terminally truncated La deletion mutants, did not depend on the reading frame of the *β-Gal* derived AUG until M(223) was deleted it was concluded that M(223) allows an internal initiation and thereby an expression of a C-terminally fragment of La protein independent of the upstream located reading frame [9]. In consequence, this C-terminal fragment starts at the same position where the suggested 25 kDa C-terminal proteolytical La protein fragment was found to start by protein sequencing [4].

In order to increase the efficiency of expression of the N-terminus in a further attempt the La protein reading frame containing the frame shift mutation was cloned into pET-3d. Now, in addition to both, the N- and C-terminal La protein fragments we obtained a protein with a molecular weight according to full length La protein. Moreover, the protein could be purified by an Nickel affinity column chromatographic step, which indicates that the C-terminal oligo(His)-tag is in the correct La protein reading frame. In addition, the full length protein reacted with both anti-La mAbs directed to the N- or the C-terminus as well as with the patient's anti-La antibody directed to the N- and C-terminus. This indicates, that at least two correct La epitope sequences must exist in the C-terminus of the expressed protein. Finally, there are a series of stop codons in the reading frame downstream of the frame shift mutation, which rule out that a 50 kDa protein can be made from this reading frame for example by overreading the first in frame stop codon.

In summary, these data allowed the conclusion that a ribosomal frame shift allowed an expression of the full length La protein from the La cDNA containing the frame shift mutation. Indeed, the long (A)-stretch of 7 (8) (A)-residues representing the hot spot region also represents a putative ribosomal frameshift site [26].

Our data show that translation when it starts at the AUG encoding for M(1) and ending 11 aa downstream of the frame shift mutation site at the first in frame stop codon results in a similar protein product of 29 kDa as it is formed when a proteolytic cleavage of full length La protein occurs in the proteolytic sensitive region. Furthermore, a reinitiation or an internal initiation at M(223) also resulted in the same C-terminal protein product of about 25 kDa as it is formed when full length La protein is cleaved within

the protease sensitive region. Therefore, at present we cannot completely rule out that the N- and C-terminal fragments present in the *E. coli* extracts and the baculovirus infected SF9 cell extracts are the result of a proteolytic degradation of the full length La protein. However, a proteolytical degradation appears rather unlikely, because an infection of SF9 with a recombinant baculovirus expressing the correct La protein reading frame resulted in an expression of full length La protein with merely traces of degradation products. In contrast, when the recombinant baculovirus containing the frame shift mutation was expresed in parallel under identical conditions, it produced nearly quantitatively N- and C-terminal La protein fragments. It is important to mention that the expression level was similar for both recombinant baculoviruses. Therefore, the appearance of the La fragments cannot be the result of a less efficient expression increasing the possibility of a degradation of the full length La protein. Having in mind that the full length La protein made from the mutant baculovirus by the use of ribosomal frame shifting should have the same primary protein sequence as the native La protein, the two La protein extracts should not differ with respect to their amount of proteolytic degradation. Thus the N- and the C-terminal La protein fragment made from the mutant recombinant baculovirus might be native translational products. In this case the translation of the C-terminal fragment required an efficient internal initiation or reinitiation at M(223) as it was found to occur in *E. coli*.

The presence of an internal reinitation site downstream of the hot spot region is also of interest from a further evolutionary point of view. Most recently, La related cDNAs were isolated from yeast [27,28]. Sequence comparisons show that only the N-terminal fragment was conserved during evolution. One possible explanation could be that the mammalian La gene is a fusion product of two genes in the hot spot region. The functions of the mammalian La protein could still be separated to two independent proteins in the yeast system. Two distinct functions had been attributed to La protein including a cytoplasmic function in internal initiation of translation and a nuclear function in transcription.

Based on the cytoplasmic localization of the N-terminal fragment and the nuclear staining of the C-terminus, one could speculate that the nuclear function is related to the C-terminus and the N-terminus could be related to the cytoplasmic function.

Both, the anti-La mAb SW5 and the patient's anti-La antibody stained predominantly the cytoplasmic compartment. As mentioned above the patient's anti-La antibody preferentially reacted with the N-terminal La protein fragment. This explains why the staining pattern of the patient's anti-La antibody was similar to the pattern of the anti-N-terminal reactive anti-La mAb SW5. As summarized in the introduction section, the N-terminal fragment contains the RNP-consensus motif, which was found to be required and sufficient for binding of La RNAs [5,6]. In consequence, the possibility to bind to RNA via this RNP-motif is not required for nuclear import and assembly with the nuclear speckles.

Finally, the identification of a ribosomal frame shift site in the hot spot region could also be involved in the formation of neoepitopes in virus infeced cells and thereby important for triggering of the autoimmune response.

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